



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Membrane pathology and microglial activation of mice expressing membrane anchored or membrane released forms of A and mutated human APP

Citation for published version:

Jeffrey, M, McGovern, G, Barron, R & Baumann, F 2015, 'Membrane pathology and microglial activation of mice expressing membrane anchored or membrane released forms of A and mutated human APP', *Neuropathology and Applied Neurobiology*, vol. 41, no. 4, pp. 458-470. <https://doi.org/10.1111/nan.12173>

Digital Object Identifier (DOI):

[10.1111/nan.12173](https://doi.org/10.1111/nan.12173)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Neuropathology and Applied Neurobiology

Publisher Rights Statement:

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/nan.12173

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Membrane pathology and microglial activation of mice expressing membrane anchored or membrane released forms of A β and mutated human APP:

M Jeffrey¹, G McGovern¹, R Barron², F Baumann³

¹ Animal Health and Veterinary Laboratories agency. Lasswade Laboratory, Pentlands Science Park, Bush Loan, Penicuik, Midlothian Scotland EH26 0PZ

² Neurobiology Division, Roslin institute & R(D)SVS, University of Edinburgh, Roslin, Midlothian, EH25 9RG

³ Department of Cellular Neurology, Hertie Institute for Clinical Brain Research, University of Tübingen. D-72076 Tübingen; and German Centre for Neurodegenerative Diseases (DZNE), D-72076 Tuebingen, Germany.

Keywords: Prion disease, glycosyl-phosphoinositol, A β , Mutant Alzheimer's Precursor Protein, microglia, protein misfolding

Running title: membrane pathology of anchored A β

The authors have no conflicts of interest with the data presented in this manuscript.

Abstract:

Aims: Alzheimer's disease and the transmissible spongiform encephalopathies or prion diseases accumulate misfolded and aggregated forms of neuronal cell membrane proteins. Distinctive membrane lesions caused by the accumulation of disease-associated prion protein (PrP^d) are found in prion disease but morphological changes of membranes are not associated with A β in Alzheimer's disease. Membrane changes occur in all prion diseases where PrP^d is attached to cell membranes by a glycosyl-phosphoinositol (GPI) anchor but are absent from transgenic mice expressing anchorless PrP^d. Here we investigate whether GPI membrane attached A β may also cause prion-like membrane lesions. **Methods:** We used immunogold electron microscopy to determine the localization and pathology of A β accumulation in groups of transgenic mice expressing anchored or unanchored forms of A β or mutated human Alzheimer's precursor protein. **Results:** GPI attached A β did not replicate the membrane lesions of PrP^d. However, as with PrP^d in prion disease, A β peptides derived from each transgenic mouse line initially accumulated on morphologically normal neurite membranes, elicited rapid glial recognition and neurite A β was transferred to attenuated microglial and astrocytic processes.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/nan.12173

Conclusions: GPI attachment of misfolded membrane proteins is insufficient to cause prion-like membrane lesions. Prion disease and murine A β amyloidosis both accumulate misfolded monomeric or oligomeric membrane proteins that are recognised by glial processes and acquire such misfolded proteins prior to their accumulation in the extracellular space. In contrast to prion disease where glial cells efficiently endocytose PrP^d to endo-lysosomes, activated microglial cells in murine A β amyloidosis are not as efficient phagocytes.

Introduction:

Alzheimer's disease (AD) and the transmissible spongiform encephalopathies or prion diseases are progressive neurological disorders in which there is a pathological accumulation of a host cell membrane protein. In AD, peptide fragments of the Alzheimer's precursor protein (APP) accumulates in the interstitial spaces as amyloid plaques while in prion disease it is a misfolded form of cell-membrane prion protein that accumulates as extracellular amyloid fibrils and amyloid plaques. Enzyme digestion of membrane spanning APP by membrane secretases leads to formation of A β ₁₋₄₀ and A β ₁₋₄₂ peptide fragments which are released to the extracellular space [1,2]. These fragments are assembled into amyloid fibrils which increasingly accumulate with age in many species and are particularly abundant as amyloid plaques in AD patients. In contrast prion protein is attached to the external leaflet of the plasma-membrane by a glycosyl-phosphoinositol (GPI) anchor [3]. Abnormal disease associated PrP (PrP^d) is a mostly full length form of the normal cell membrane protein which has undergone a putative conformational transition into an abnormal and pathological isoform [4]. This PrP^d may accumulate on membranes or be released to the extracellular spaces where it may also aggregate in the form of amyloid fibrils or plaques [5,6].

Although extracellular aggregates of PrP^d or A β fibrils are abundant in brains of individuals with prion diseases and AD patients respectively, the precise contribution each of these protein aggregates may make towards neurological deficits is unclear. Nevertheless both PrP^d and A β may cause alterations of neuronal membranes which may contribute to neuronal dysfunction. GPI anchored PrP^d is closely associated with a group of highly distinctive morphologic changes in neurite membranes (for review see [6]). PrP^d monomers or oligomers appear to be poorly internalized from neuronal membranes which results in abnormal membrane recycling of a ubiquitinated PrP^d complex with formation of bizarre coated and spiral membrane invaginations, sub-membrane cisterns and excess coated pits [6]. Morphological changes of membranes are not reported in AD patients nor are they reported in murine transgenic models of A β amyloidosis, albeit other studies have suggested that oligomeric A β aggregates may have molecular effects on membranes leading to

alterations to the biophysical structure of the lipid bilayer, ion channels and conductivity [7,8,9,10].

The membrane changes associated with PrP^d are found in all species examined including mice and in most prion strains [6] with the notable exception of scrapie infected transgenic mice expressing anchorless PrP. A GPI deficient prion protein molecule has been generated in a transgenic mouse and in which cellular PrP is not retained on the cell membrane [11]. Following infections with a murine adapted sheep scrapie prion strain, such mice do not show abnormal membrane changes but increased plaques, and cerebral amyloid angiopathy [11]. These data suggest that GPI attachment of aggregated or pathological prion protein to membranes may be important for generation of morphologic membrane lesions. A GPI anchored A β molecule has been generated in a transgenic mouse line which is also associated with increased plaque formation relative to non-anchored A β [12].

In this study we used immuno-electron microscopy to determine whether a GPI anchored A β peptide would cause morphological membrane lesions similar to those observed in prion disease. The results show that GPI membrane linked A β does not replicate the membrane lesions of PrP^d. However, we observed that pathological anchored and anchorless A β , in common with PrP^d of prion diseases, both initially accumulate on neurite membranes. Such membrane associated A β is rapidly recognised by microglia and astrocytes which show corresponding morphologic change and acquire these A β aggregates of neuritic origin prior to their release and aggregation in the extracellular space.

Materials and Methods.

Experimental animals:

All mice were kept under specific pathogen-free conditions. The experimental procedures were carried out in accordance with the veterinary office regulations of Baden-Wurttemberg (Germany) and all experiments were approved by the Institutional Animal Care and Use Committees.

Whole brains from eight transgenic mice were perfused fixed with 4% paraformaldehyde / 0.1% glutaraldehyde. The transgenes expressed by the mice corresponded to co-expression of anchored A β and mutant APP at 15 months of age (n=2; A β eGPI x APP23); co-expression of anchorless A β and mutant APP at 13 months of age (n=2; A β e x APP23); co-expression of anchored A β and mutant APP (n=2; A β eGPI x APP23) and expression of a mutant APP alone at 13 months of age (n=2 ; APP23). A β eGPI mice express an A β ₁₋₄₂ peptide attached to a GPI moiety of the PrP protein while A β e mice release A β ₁₋₄₂ without the GPI anchor. APP23 mice express a human APP containing the Swedish mutation (670/671_{KM-NL}). Further details of the generation of these mice and the evidence for increased plaque formation in A β eGPI x APP23 mice can be found in Nagarathinam et al. [12]. Quantitative assessment of plaque numbers or density was not performed in the

present study. J20 mice which over-express double human Swedish and Indiana APP mutations (670/671^{KM-NL}; and 717^{V-F}) [13] as well as wild-type mice and anchorless PrP transgenic mice [11] both of which express full length murine APP, were also available as additional positive and negative controls.

Methods:

Brains of each mouse were coronally sectioned at 1mm intervals and alternate 1mm tissue slices were embedded in paraffin wax. 1mm cubes were sampled from the remaining sections to obtain representation of thalamus, hippocampus, cerebral cortex and cerebellum, and were embedded in araldite resin.

Immunohistochemistry and immunoblotting

The light microscopical immunohistochemical procedure was used as described previously [14]. 5µm sections were labeled for Aβ₁₋₄₂ using antibody AHP677 (AbD Serotec, Oxford, UK) at a 1:500 dilution, and for Aβ₁₋₄₀ using AHP676 (AbD Serotec) and ABN 240 (Millipore UK limited, Watford, UK) at 1:500 and 1:100 dilutions respectively. Immunolabeling was visualised using the avidin-biotin method (Vector Labs, Peterborough, UK) and 3,3'-Diaminobenzidine reaction product. None of these antibodies distinguish between the GPI anchored Aβ peptide or Aβ peptides derived from the APP23 transgene.

We further tested the specificity of antibody AHP 676 by dot blotting and Western blotting. AHP676 was unable to discriminate between Aβ₁₋₄₀ and Aβ₁₋₄₂ peptides (data not shown).

Light Microscopy procedure – Resin

The avidin-biotin complex immunohistochemical staining method was applied to the etched and pre-treated 1µm sections using all Aβ antibodies mentioned above. Selected tissue blocks with appropriate immunolabelled areas and control blocks were then taken for sub-cellular analysis. Multiple sections from at least two blocks from each brain area were studied from each animal.

Ultrastructural immunohistochemical procedure

Resin embedded tissue blocks that contained representation of diffuse Aβ labelling and Aβ plaques were trimmed to mesas and sections cut at 60nm and labeled as described previously [15]. Briefly, sections were taken from resin blocks previously found to contain plaques, and were labelled using immunogold methods with AHP677 and AHP676 at a 1:10 dilution or ABN 240 at a 1:20 dilution. Labelling was maximised by using a formic acid pre-treatment step, however, this compromises tissue structure. In the absence of formic acid, tissue structure is optimal though some immune signal is lost. Tissue sections were analysed both with and without formic acid pretreatment. All illustrations are from non-formic acid treated samples. Tissues were also counterstained with uranyl acetate and lead citrate (UA/LC).

In an earlier study Nagarathinam et al [12] showed both by biotinylation and by immunofluorescence that the Aβ_eGPI anchored peptide, but not the Aβ_e unanchored

peptide, reaches the surface of transfected HEK cells from where it may be released by cleavage of the GPI anchors using Phospholipase C. These data confirm that the A β eGPI peptide attaches to the cell surface. However, it is important to note that the antibodies used do not permit discrimination between peptides produced in association with the APP23 allele and the A β eGPI or A β e alleles. In co-expressing mice we cannot therefore be certain whether individual immunogold labeled A β deposits are anchored or unanchored.

In the previous studies of anchored or anchorless A β mice Nagarathinam et al., [12] showed that the transgenic A β eGPIxAPP23 line accumulated more A β than did the transgenic A β exAPP23 line. Measuring the frequency or abundance of products using electron microscopy is difficult and requires rigorous stereotactic sampling. We did not sample with a view to performing morphometric studies and we made no evaluation of the relative amounts of A β detected in each model system. Our observations therefore provide no judgment on whether the kinetics of GPI- or GPI+ A β molecular assembly is more or less efficient.

Results:

A β antibodies detect only pathological A β in transgenic mice.

In the present study we used A β e and A β eGPI mice intercrossed with APP23 mice. While A β eGPI mice express an anchored version of A β liberated from a Bri A β eGPI precursor protein, A β e mice liberate from a similar precursor an eight amino-acid extended non-anchored A β . APP23 mice over-express human APP with the Swedish mutation (670/671_{KM-NL}) resulting in an overproduction of human A β ₁₋₄₀ and A β ₁₋₄₂. In previous studies mice expressing anchored or anchorless A β peptide displayed no plaque accumulations or other plaque associated pathology up to 24 months of age. However, when such mice were crossed with mutant human APP expressing mice, the mice that expressed anchored A β peptide showed enhanced plaque pathology when compared with mice expressing anchorless A β [12].

Each of the A β ₁₋₄₀ or A β ₁₋₄₂ antibodies (Fig 1 b,c) readily detected plaques both in wax or plastic embedded tissue sections. The cores of plaques were more weakly labeled than the intensely labeled radiating bundles of amyloid extending from these cores (Fig 1a,b,c). APP23 and double transgenic A β eGPI x APP23 mice showed marked A β accumulation as amyloid plaques in a primarily cerebro-cortical distribution as previously described [16]. J20 mice expressing the double human Swedish and Indiana mutations showed a similar distribution of plaques also as previously described [16].

In addition to plaques, A β ₁₋₄₀ or A β ₁₋₄₂ antibodies also detected intracytoplasmic labelling which in 1 μ m thick plastic embedded sections presented as granular or globular labelling within glial cytoplasm or neuronal cytoplasm. Intraglial labelling was most frequently found at the periphery of plaques (Fig 1c) and, although intra-neuronal labelling was present throughout the cerebral cortex and hippocampus, the frequency of intra-neuronal labelling was also considered to be more frequent in neuropil surrounding plaques (Fig 1c).

No differences in ultrastructural immunolabelling patterns were observed when antibodies to A β ₁₋₄₀ were compared with antibodies to A β ₁₋₄₂.

No A β labelling was detected with A β ₁₋₄₀ or A β ₁₋₄₂ antibodies in murine APP expressing wild-type mice or in anchorless PrP control tissues. Numerous blocks taken from brain regions of A β transgenic mice that lacked plaques (such as thalamus) were also unlabelled. This suggests that the combination of fixation in mixed aldehydes and embedding in plastic permits detection of only pathologically altered human A β by the antibodies used.

A β is present at three sub-cellular locations in transgenic tissues

In each of the transgenic A β mice three types of A β accumulation were detected. Firstly, fibrillar aggregates of A β could be recognized in both immunogold labeled and UA/LC stained sections as mature classical amyloid plaques composed of variously sized bundles of amyloid fibrils (Fig 2a,b) or as individual small and randomly orientated filaments located in the extracellular space (Fig 2e). The latter occurred at the periphery of mature plaques and also as apparently immature or as nascent plaques. Secondly, non-fibrillar membrane associated accumulations of A β (Figs 3,4) were abundant and was found in all transgenic mice irrespective of the nature of the transgenic APP mutation or putative presence or absence of GPI anchors. Membrane A β derived from mutant APP23 and J20 mice may potentially occur within membranes while that of GPI anchored A β would be expected to occur at the exterior surface of the plasma-membrane. The immunogold methods used here have an accuracy of approximately 18.5 nm relative to their A β molecular target [17] and as membranes are 9nm thick we were therefore unable to determine whether the membrane topology of A β accumulating in APP23, J20 A β e x APP23 mice was the same as, or different from A β eGPI x APP23 mice expressing GPI anchored A β . Thirdly, non-fibrillar intracellular intra-endo-lysosomal A β accumulations (Fig 5) could be visualized in neurons, astrocytes and microglia using immunogold localization methods but were not otherwise appreciable in heavy metal stained sections.

Neither membrane associated nor extracellular A β is associated with morphologic membrane change.

A β amyloid plaques had the typical morphological features of these structures as previously described in man and rodents [16,18]. Briefly, plaques consisted of dense cores composed entirely of closely packed interweaving bundles of amyloid fibrils which excluded other cytological elements (Fig 2a) and fibrils were strongly labeled with antibodies to both A β ₁₋₄₀ (Fig 2b) and A β ₁₋₄₂ (Fig 2e). Bundles of amyloid fibrils extended from these cores (Fig 2b) and were surrounded by dystrophic neurites, microglial or astrocytic cytoplasm (Fig 2a). Bundles of amyloid fibrils gradually attenuated to small clusters of fibrils and finally individual fibrils between neurites (Fig 2e). Microglial cytoplasm was often deeply indented by bundles of amyloid (see

Fig 5 a,b). At the margins of plaques, where amyloid fibrils were no longer visible, processes and synaptic structures were ensheathed and isolated by microglia or astrocytic cytoplasm. Rarely amyloid plaques were found in the meninges (not shown) and amyloid fibrils were observed within basement membranes of blood vessels (Fig 2 c,d). Neither extracellular bundles of amyloid fibrils nor individual fibrils were associated with morphological changes of adjacent neuritic membranes.

Numerous foci of A β immunogold labelling were found in areas of neuropil lacking bundles or individual amyloid fibrils. Rarely, such foci of A β immunolabelling were exclusively associated with the membranes of small diameter neurites: small dendrites and dendritic spines, small unmyelinated axons and axon terminals (Fig 3a-e). These occurred in the absence of fibrils or of reactive glial processes or of any conspicuous change to the morphology of neuritic membranes (Fig 3a-e). Such foci were present in transgenic mice expressing double (J20) (Fig 3 a,b) or single human mutations (APP23) of APP (Fig 3c) as well as in mice expressing unanchored (A β e x APP23) (Fig 3 d) or anchored (A β eGPI x APP23) (Fig 3e) forms of A β . We interpreted this as the first sites and forms of A β aggregation or accumulation on or in membranes.

Glial cells rapidly recognize and respond to membrane associated A β

Some foci of neuritic membrane immunolabelling were additionally associated with the presence of numerous thin glial processes interweaving between neurites and sometimes encircling individual processes (Fig 4a). These foci were many times more numerous than those in which neuritic membrane associated A β accumulation was confined to neurites alone. Although both astrocytic and microglial processes were involved, microglial cells were more conspicuous and more frequent than astrocytic processes (Fig 4b). Around some neurites the microglial processes that lay between them contained the immunogold label while the adjacent neuritic membranes were not labeled or showed a low level of labelling (Fig 4 b,c,d). We consider this represents a sequence of pathological processes: the initial event observed by immunogold electron microscopy is accumulation of misfolded A β on neurites. Newly formed misfolded membrane A β is recognized quickly by glia, particularly microglia which infiltrate and surround A β accumulating neurites. A proportion of neuritic membrane A β is transferred to these contacting microglial or astrocytic processes. Similar neuritic membrane and microglial A β labelling were also evident at the margins of plaques where no amyloid fibrils were evident. No morphologic change of membranes was recognized in association with this A β accumulation and glial infiltration (Fig 4 a-e).

Intra-cellular A β is located in lysosomes

Endo-lysosomes of microglia (Fig 5a,b,c), astrocytes (Fig 5d) and neurons (not shown) were labeled for A β . Lysosomes containing A β were often enlarged (Fig 5c) and lysosomes were often more numerous in those microglia that had intracellular A β . By electron microscopy examination only a small fraction of neurons showed intra-lysosomal labelling (not shown). Similarly, most glial cells did not show

evidence of intra-cellular A β labelling and where intra-lysosomal labelling was observed it occurred in only a fraction of the lysosomes present. Intra-lysosomal labelling of microglia was most frequently observed around mature amyloid plaques and was not seen in areas where immunolabelling was confined to membranes.

A β transgenes have no effect on the sub-cellular locations of A β

We were unable to recognize qualitative morphological differences between any of the transgenic lines examined. In each transgenic mouse examined all three patterns of amyloid, pre-amyloid (Fig 3a-e) and intracellular A β labelling and fibrillisation were seen. Similarly early microglial associations with membrane A β were observed also in each mouse line examined (Fig 4 a-e). Vascular amyloid was identified in all mice including the A β eGPIxAPP23 but was most frequently identified in the double APP mutated J20 mouse. Additionally linear accumulations of A β appearing on larger dendrites (Fig 3b) were also more conspicuous in this mouse.

Discussion:

In none of the four lines of transgenic mice modeling A β amyloidosis examined in the present study was there evidence of membrane deformation such as is associated with PrP^d accumulation in prion diseases [6]. As shown previously, compared to the co-expressed APP derived A β , A β eGPI accounts only for one tenth of the total amount of A β in the young pre-depositing mice at the age of 2 months [12]. This ratio is preserved even after deposition of amyloid plaques at 9 months in the double transgenic A β eGPI x APP23 mice and remains on this level. Thus, though anchored A β cannot be specifically detected by our immunogold labelling, its presence can be inferred and therefore the absence of any morphologic changes of neuritic membranes seen in A β eGPI x APP23 mice indicates that GPI attachment of aggregated peptides is not sufficient to cause morphologic changes of neurite plasma-membranes. While we cannot exclude the possibility that more conspicuous membrane lesions may have been observed had A β eGPI been present in greater abundance, we nevertheless provisionally conclude that properties intrinsic to PrP^d are necessary to cause increased coated pits, membrane invagination and spiral deformation of membranes and that the aggregation of other proteins on or in membranes does not invariably result in morphological changes associated with dysregulation of endocytosis.

Pre-amyloid forms of A β were recognized on membranes in each transgenic mouse line. This suggests that peptides formed in A β eGPI x APP23 or A β e x APP23 mice, as well as A β peptides arising from single or double mutated human APP, were each able to accumulate on or in membranes and that this membrane association (putatively membrane assisted aggregation) is a necessary step in the assembly of A β monomers prior to their release and quaternary organization into fibrils within the interstitial spaces. In previous electron microscopy studies of 3xTg-AD mice and aged dogs [19], 3D reconstruction of immunolabelled thin sections showed membrane associated A β arranged in “sheets and tendrils” on soma and large dendrites suggesting a precise linkage of A β to other molecular components of dendrosomatic

membranes. In agreement with this study we found a significant fraction of pre-fibrillar A β was intimately associated in two dimensions with linear segments of plasma-membranes of large dendrites in the J20 mice (Fig 3d). However, in contrast with the observations of Nuntagij et al., [19] we did not find conspicuous linear large dendrite associated A β accumulation in the APP23 mice or in the anchored or non-anchored A β crosses. In each of these models a significant fraction of membrane A β accumulation or aggregation was found as individual immunogold label on small diameter dendrites, axon terminals and also in lysosomes and in association with microglial processes. The reasons for these differences may be technical (different antibodies and post-embedding immuno-methods were used) or due to differences in the molecular constructs and species of the study subjects.

Since the initial descriptions of microglia surrounding amyloid plaques, the microglial-mediated inflammatory response in AD has been extensively studied [19,20]. The phenotype of activated microglia in AD and in murine models of A β amyloidosis are generally considered benign and aimed at clearing damaged tissue with minimal further disruption [20,21,22] and microglia are apparently neither necessary or sufficient to form or maintain plaques [23]. Microglia in a non-activated state, or expressing pro-inflammatory cytokines appear unable to degrade internalized A β while those experimentally induced to express anti-inflammatory cytokines promote degradation *in vivo* [24,25,26]. It is postulated that microglia may contribute to disease progression by failing to switch to an adaptive or anti-inflammatory response. Results of several studies have suggested that the initial immune response, when plaques and tangles are recognized as invading stimuli, may be inefficiently followed by an adaptive or anti-inflammatory immune response [20,21,27]. The present study provides evidence for an early microglial response before release of A β to the extracellular space.

Although we did not examine mice at early stages of disease, we can nevertheless infer certain stages in the formation of plaques in mice killed at 13 and 15 months of age. A β was immunolocalised to the membranes of small neurites in the absence of any morphological change indicating this to be the earliest stage of pathology we could observe in the electron microscope. Foci of membrane associated A β accumulation also showing infiltration by microglial or astrocyte processes between neurites were much more numerous than foci of neurite A β accumulation alone. This suggests that the initial retention of peptide monomers or small multimers of A β within the membrane was readily detected by glial cells and in particular microglia. Microglia processes in these foci are numerous and often showed increased electron density. Some foci showing A β within microglial cytoplasm in the absence of neuritic membrane A β suggests that neurites transfer these initial A β deposits from their plasma-membranes to microglial and astrocytic processes.

Many published AD studies focus on the activation of microglia in response to plaques and to fibrillar forms of A β . As CNS representatives of the innate immune system response, microglia express Toll-like receptors which are able to detect microbial and viral pathogens and tissue damage [24, 27]. In AD, Toll-like receptors

are thought to be necessary for recognition and clearance of higher weight A β species. However, Toll-like receptors have evolved to recognize intracellular proteins and pathogen associated molecular patterns and it has generally been thought that monomeric and oligomeric species of A β were not recognized by these receptors. We have observed that microglia recognize and show morphologic changes when pathologic membrane associated monomeric or oligomeric species of pre-fibrillar A β are present on neurites. The apparent rapid response of microglia to cell surface A β is in agreement with imaging studies showing how the processes of so-called resting microglia are in continuous motion, constantly monitoring their microenvironment and are in contact the surface of all nearby cellular elements every few hours [28]. The cellular location of plasma-membrane A β on microglia are somewhat at variance from previous studies in which it has been suggested that soluble A β is internalized through constitutive non-saturable fluid phase macropinocytosis [29]. In our studies we did not see evidence of pinocytosis, rather, in each of the murine transgenics, A β is apparently passively transferred to microglia plasma-membranes from neuritic membranes at these earliest stages of accumulation. GPI anchored proteins are capable of passive transfer from one membrane to another [30] as also occurs between membranes accumulating PrP^d in prion diseases [6,31]. In the present study no difference was found for this putative membrane transfer for anchored or non-anchored A β .

Intracytoplasmic A β was observed in a proportion of microglia, particularly those surrounding mature plaques. Lysosomal A β was found at a very much lower frequency than we observed for PrP^d in prion disease infected brain [6] where lysosomal PrP^d is frequently observed in areas of membrane PrP^d accumulation lacking fibrils [32]. A major difference between prion diseases and the transgenic A β mice studied above is the protein expression by glial cells. Mouse APP is naturally expressed by murine glial cells but A β eGPI, A β e peptides and human APP transgenes are all expressed by the Thy1 promoter [12] and mutant APP in J20 mice is controlled by platelet-derived growth factor β [13]. Thus A β derived from transgenes will be absent or minimally expressed by glia while in prion diseases, prion proteins are constitutively expressed by glial cells. The activation of microglia and transfer of PrP^d to glial cells might therefore be aided by the expression of prion protein at the cell surface of glia, but A β inhibited from transfer by deficiency of mutant APP on microglia and astrocytes. However, microglia that lack prion protein in a transgenic line where prion protein was controlled by the glial fibrillary acidic protein promoter on a null mouse background, are also capable of taking up PrP^d [31]. This suggests that the presence of the corresponding normal prion protein or A β peptide on microglia is not required for the recognition and transfer of misfolded proteins by glial cells. We therefore suggest that microglia are not as efficient at internalising membrane associated A β when compared to membrane PrP^d.

In summary, this study has shown that the membrane pathology of prion disease was not replicated in the A β eGPI x APP23 mouse, albeit membrane attached forms of A β are present in low abundance in this mouse, and we therefore suggest that the membrane changes seen in prion disease affected tissues are not non-specific responses to membrane aggregation of GPI anchored membrane proteins. However,

our studies show that the glial responses to neurite membrane protein misfolding are similar in both prion disease and murine models of A β amyloidosis. In common with membrane misfolding associated with PrP^d, these studies suggest that microglia and also astrocytes recognize and react to A β while still present on or in plasma-membranes and that such recognition are not dependent upon its release to the extracellular space or its assembly into quaternary fibrillar structures. Glial cell responses to non-fibril membrane forms of pathologic A β are irrespective of the original APP mutation or of its GPI directed localization on the membrane. As with PrP^d in prion disease, membrane A β appears to physically transfer to membranes of microglia but, unlike in prion disease, microglia do not as readily internalize A β to lysosomes.

Acknowledgements: MJ, FB and GMcG conceived and designed the study; FB and RB carried out animal studies; MJ, GMcG, FB prepared and characterised reagents and carried out pathology: MJ wrote the paper with contributions by FB, GMcG, and RB. We are grateful to the Gladstone Institute of Neurological disease University of California for provision of J20 mice and to Callum Donnelly for skilful technical assistance. Roslin Institute is supported by Institute Strategic grant funding from BBSRC.

Legends for Figures

Figure 1: A β ₁₋₄₀ and A β ₁₋₄₂ antibodies label amyloid plaques and intracellular accumulations of A β in plastic embedded tissue blocks.

- A β ₁₋₄₂ accumulation as amyloid plaques in the cerebrum of an A β e x APP23 mouse. Antibody AHP 677. Bar= 200 μ m.
- Higher magnification of (a) showing the cores of plaques have a lower labelling intensity as compared with their periphery. Presumed intracellular A β ₁₋₄₂ in microglia (arrowheads) and neuronal perikaryonal cytoplasm (arrow) is infrequently present around this group of plaques. Antibody AHP 677. Bar= 50 μ m.
- A β ₁₋₄₂ accumulation as amyloid plaques in the cerebrum of an A β eGPI x APP23 mouse. The plaque also shows lesser intensity of labeling in the core and is surrounded by occasional intracytoplasmic microglial (arrowheads) and intracytoplasmic neuronal (arrow) A β accumulation. Antibody AHP 677. Bar= 50 μ m.

Figure 2 : A β accumulates as amyloid plaques and extracellular amyloid fibrils in transgenic mice expressing mutant APP.

- Mature plaque from 13 month old A β eGPI x APP23 mouse showing central core of dense amyloid surrounded by dystrophic neurites (d). UA/LC Bar = 5 μ m

- b) A β_{1-40} immunogold labeled bundles of amyloid fibrils from the periphery of a mature plaques in an APP 23 mouse. Antibody: ABN240, bar = 2 μ m.
- c) A β_{1-40} immunolabelling associated with the basement membranes of a small arteriole in a J20 mouse. Antibody: AHP676, bar = 2 μ m.
- d) A β_{1-42} immunolabelling associated with the basement membranes of a small arteriole in an APP 23 mouse. Fibrils are just discernable within the basement membrane (arrows) . Antibody: AHP677, bar = 2 μ m.
- e) A β e x APP 23 mouse. At the periphery of mature plaques individual fibrils and small bundles of fibrils are immunolabelled for A β_{1-42} between viable neurites (n) and microglial processes (mg). Antibody: AHP677, bar = 500nm.

Figure 3: A β labelling occurs on plasma-membranes of small neurites, including terminal axons, that are otherwise morphologically normal and without infiltration of glial processes or extracellular amyloid.

- a) A β_{1-42} immunogold labelling of the plasma-membranes of small diameter neurites in a J20 mouse (axt =axon terminal). Antibody: AHP677, bar = 500nm
- b) As above but one process sectioned longitudinally shows labelling along the length of the plasma membrane (between arrowheads). Antibody: AHP677, bar = 500nm.
- c) APP23 mouse showing A β_{1-40} labelling of the plasma-membrane of an axon terminal (axt) containing synaptic vesicles. Antibody: ABN240, bar = 500nm.
- d) A β_{1-42} labelling associated with the plasma-membrane of small diameter neurites (probable unmyelinated axons and axon terminals) of an A β e x APP23 mouse. An un-reactive astrocyte process is also present (as). Antibody: AHP677, bar = 500nm.
- e) A β eGPI x APP 23 mouse showing A β_{1-40} labelling associated with axon terminal(axt) and other neurite plasma-membranes (arrowheads). Antibody: ABN240, bar = 500nm.

Figure 4: Prominent microglial processes are associated with neurite membrane accumulation of A β in the absence of extracellular fibrils.

- a) J20 mouse showing thin microglial processes (arrows) surrounding and isolating morphologically normal neurites. UA/LC, bar = 500nm.
- b) APP23 mouse showing prominent A β_{1-40} associated with dark microglial cytoplasm (arrows). A β_{1-40} labelling is present both on membranes of neurites and microglial processes. Antibody: ABN240, bar = 500nm.

- c) APP23 mouse showing microglial cytoplasm inserted between neurites. Clusters of $A\beta_{1-42}$ immunolabelling can be visualized in association with microglial processes and membranes of neurites. Within some clusters individual immunogold label can be seen on neurite membranes (arrowheads) and others on microglial processes (arrows). Antibody: AHP677, bar = 500nm.
- d) A β eGPI x APP23 mouse with a similar field to that shown above. Immunogold label for $A\beta_{1-40}$ is associated with both neurite membranes (arrowheads) and thin glial processes running between neurites (arrows). The irregular cross sectioned process without organelles (as) is probably an astrocytic process. ax: axon terminal; ds dendritic spine. Antibody: ABN240, bar = 500nm.
- e) A β e x APP23. An axon terminal (axt) is associated in approximately half of its circumference with $A\beta_{1-42}$ labelling. The labelling is within the adjacent extracellular space where very fine glial processes are just evident (arrow heads) and on the limiting plasma membrane. Antibody: AHP677, bar = 500nm.

Figure 5: $A\beta$ labelling occurs in a proportion of glial lysosomes.

- a) A β e x APP23 mouse showing bundles of immunogold labeled $A\beta_{1-42}$ amyloid entirely surrounded by microglial cytoplasm. A large irregular lysosome (arrow) in adjacent microglial cytoplasm is also labeled for $A\beta_{1-42}$. Antibody: AHP677, bar = 1 μ m.
- b) A β eGPI x APP23 mouse also showing bundles of immunogold labelled $A\beta_{1-40}$ engulfed by microglial cytoplasm. A small ovoid endo-lysosome (arrow) in the adjacent cytoplasm is also labeled for $A\beta_{1-40}$. Antibody: ABN240, bar = 1 μ m.
- c) A β eGPI x APP23 anchored mouse with a large lysosome heavily labelled with $A\beta_{1-40}$. The labelling is not uniformly distributed within the lysosome. Antibody: ABN240, bar = 1 μ m.
- d) A β eGPI x APP23 anchored mouse showing an astrocyte with three lysosomes lightly labeled for $A\beta_{1-42}$. Antibody: AHP677, bar = 1 μ m

References:

- 1 De Strooper B, Iwatsubo T, Wolfe MS. Presenilins and γ -secretase: structure, function and role in Alzheimer's disease. *Coldspring Harb Perspect Med* 2012; a006304
- 2 Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis J-C, Collins F, Treanor J, Rogers G, Citron M. β -Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* 1999; 286: 735-41
- 3 Stahl N, Baldwin MA, Burlingame AL, Prusiner SB. Identification of glycoinositol phospholipid linked and truncated forms of the scrapie prion protein. *Biochem* 1990; 29: 8879-8884
- 4 Prusiner SB, Peters P, Kaneko K, Taraboulos A, Lingappa V, Cohen FE, DeArmond SJ. Cell biology of prions. In Prion Biology and Diseases Ed SB Prusiner. New York: Cold Spring Harbor Laboratory Press. 1999: 349-391
- 5 Jeffrey M, Goodsir CM, Bruce M E, McBride PA, Scott JR, Halliday WG. Infection specific prion protein (PrP) accumulates on neuronal plasmalemma in scrapie infected mice. *Neurosci Lett* 1992; 147: 106-109
- 6 Jeffrey M, McGovern G, Siso S, González L. Cellular and sub-cellular pathology of animal prion diseases: relationship between morphological changes, accumulation of abnormal prion protein and clinical disease. *Acta Neuropathol* 2011; 121: 113-134
- 7 Hebda JA, Miranker AD. The interplay of catalysis and toxicity by amyloid intermediates on lipid bilayers: insights from type II diabetes. *Ann Rev biophys* 2009; 38: 125-152
- 8 Sokolov Y, Kozak JA, Kaye R, Chanturiya A, Glabe C, Hall JE. Soluble amyloid oligomers increase bilayer conductance by altering dielectric structure. *J Gen Physiol* 2006; 128: 637-647
- 9 Stefani M. Structural features and cytotoxicity of amyloid oligomers: implications in Alzheimer's disease and other diseases with amyloid deposits. *Prog neurobiol* 2012; 99: 226-245
- 10 Williams TL, Serpell LC. Membrane and surface interactions of Alzheimer's A β peptide-insights into the mechanism of cytotoxicity. *FEBS J* 2011; 278: 3905-3917
- 11 Chesebro B, Race B, Meade-White K, LaCasse R, Striebel J, Klingeborn M, Race R, McGovern G, Dorward D, Jeffrey M. Fatal transmissible amyloid

encephalopathy: a new type of prion disease associated with lack of prion protein membrane anchoring. *Plos Path* 2010; 6: e1000800

12 Nagarathinam A, Hoflinger P, Buhler A, Schafer C, McGovern G, Jeffrey M, Staufenbiel M, Jucker M, Baumann F. Membrane anchored GPI negative Amyloid Beta accelerates amyloid formation and exacerbates amyloid-associated toxicity in mice. *J Neurosci* 2013; 33: 19284-19294

13 Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L. High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J Neurosci* 2000; 20: 4050-4058

14 González L, Martin S, Houston FE, Hunter N, Reid HW, Bellworthy SJ, Jeffrey M. Phenotype of disease-associated PrP accumulation in the brain of bovine spongiform encephalopathy experimentally infected sheep. *J Gen Virol* 2005; 86: 827-838

15 McGovern G, Brown KL, Bruce ME, Jeffrey M. Murine scrapie infection causes an abnormal germinal centre reaction in the spleen. *J Comp Pathol* 2004; 130: 181-194

16 Terry RD, Gonata NK, Weiss M. Ultrastructural studies in Alzheimer's presenile dementia. *Am J Pathol* 1964; 44: 269-97

17 McGovern G, Mabbott N, Jeffrey M. Scrapie affects the maturation cycle and immune complex trapping by follicular dendritic cells in mice. *PLoS ONE* 2009; 4: e8186

18 Wegiel J, Wisniewski HM. The complex of microglial cells and amyloid star in three-dimensional reconstruction. *Acta Neuropathol* 1990; 81: 116-124

19 Nuntagij P, Oddo S, LaFerla FM, Kotchabhakdi N, Ottersen OP, Torp R. Amyloid deposits show complexity and intimate spatial relationship with dendrosomatic plasma membranes: an electron microscopic 3D reconstruction analysis in 3xTg-AD mice and aged canines. *J Alzheimers Dis* 2009; 16: 315-23

20 Perry VH. Contribution of systemic inflammation to chronic neurodegeneration. *Acta Neuropathol* 2010; 120: 277-286

21 Zotova E, Nicoll JAR, Kalaria R, Holmes C, Boche D. Inflammation in Alzheimer's disease: relevance to pathogenesis and therapy. *Alz Res Ther* 2010; 2: 1-9

22 Stalder M, Phinney A, Probst A, Sommer B, Staufenbiel M, Jucker M. Association of microglia with amyloid plaques in brains of APP23 transgenic mice. *Am J Pathol* 1999; 154: 1673-1684

23 Grathwohl SA, Kalin RE, Bolmont T. Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia. *Nat Neurosci* 2009; 12: 1361-1363

24 Lee CYD, Landreth GE. The role of microglia in amyloid clearance from the AD brain. *J Neur Trans* 2010; 117: 949-960

25 Majumdar A, Cruz D, Asamoah N, Buxbaum A, Sohar I, Lobel P, Maxfield FR. Activation of microglia acidifies and leads to degradation of Alzheimer's amyloid fibrils. *Mol Biol Cell* 2007; 18: 1490-1496

26 Paresce DM, Chung H, Maxfield FR. Slow degradation of aggregates of the Alzheimer's disease amyloid beta-protein by microglial cells. *J Biol Chem* 1997; 272: 29390-29397

27 Cameron B, Landreth GE. Inflammation, microglia and Alzheimer's disease. *Neurobiol dis* 2010; 37: 503-509

28 Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 2005; 308: 1314- 1318

29 Mandrekar, S, Jiang Q, Lee CY, Koenigsnecht-Talboo J, Holtzman Dm, Landreth GE. Microglia mediate the clearance of soluble fluid phase macropinocytosis. *J Neurosci* 2009; 29: 4252-4262

30 Ilangumaran S, Robinson PJ, Hoessli DC. Transfer of exogenous glycosylphosphatidylinositol (GPI)-linked molecules to plasma membranes. *Trends Cell Biol* 1996; 6: 163-167

31 Jeffrey M, Goodsir CM, Race RE, Chesebro B. Scrapie-specific neuronal lesions are independent of neuronal PrP expression. *Ann Neurol* 2004; 55: 781-792

32 Jeffrey M, McGovern G, Goodsir CM, González L. Strain-associated variations in abnormal PrP trafficking of sheep scrapie. *Brain Pathol* 2009; 19: 1-11

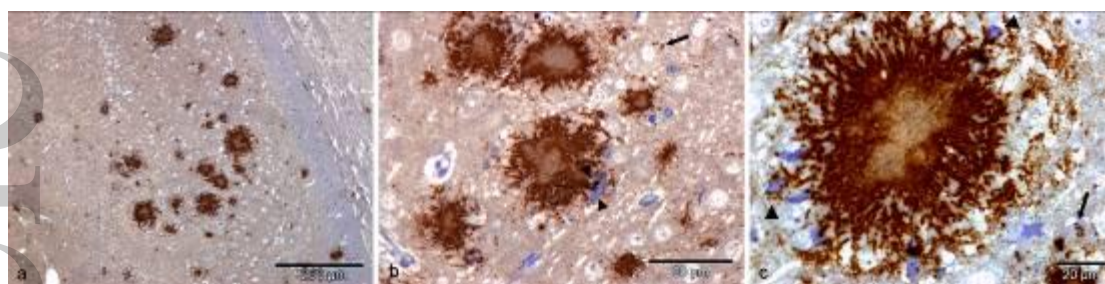


Figure 1

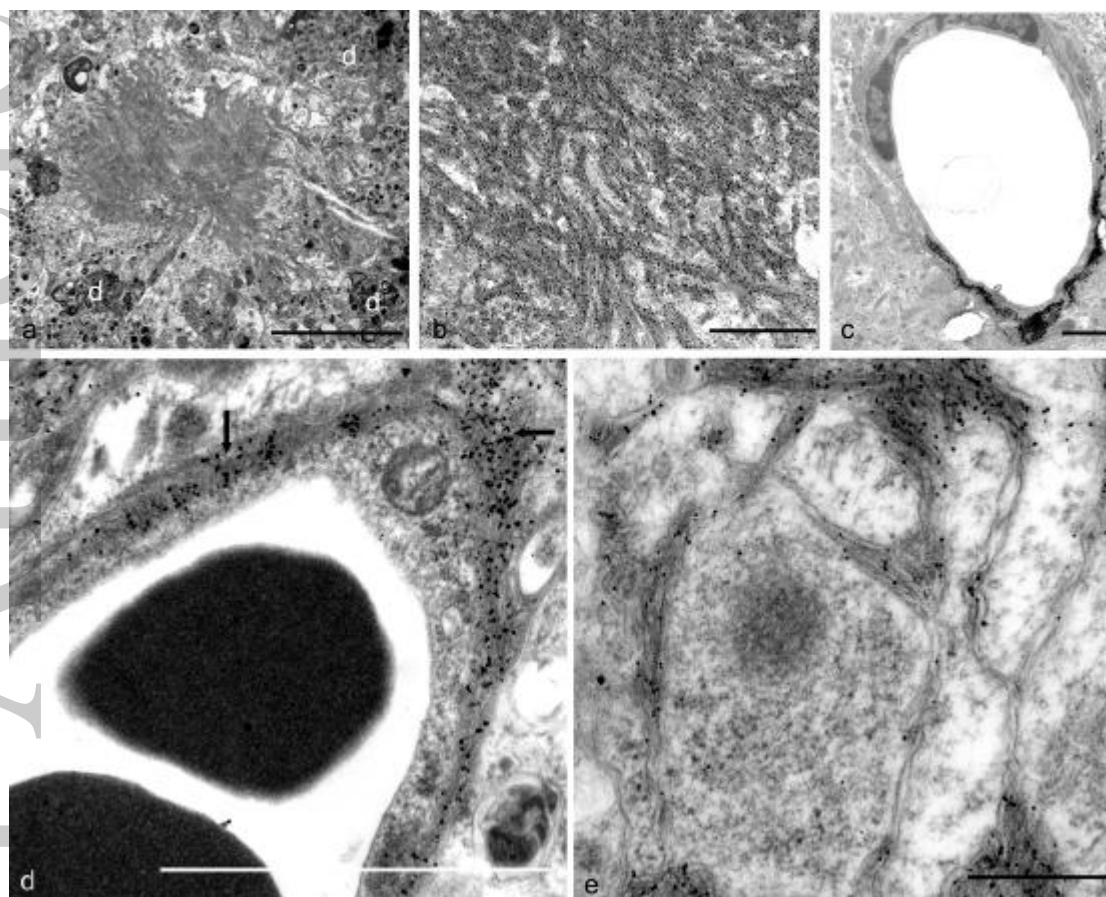


Figure 2

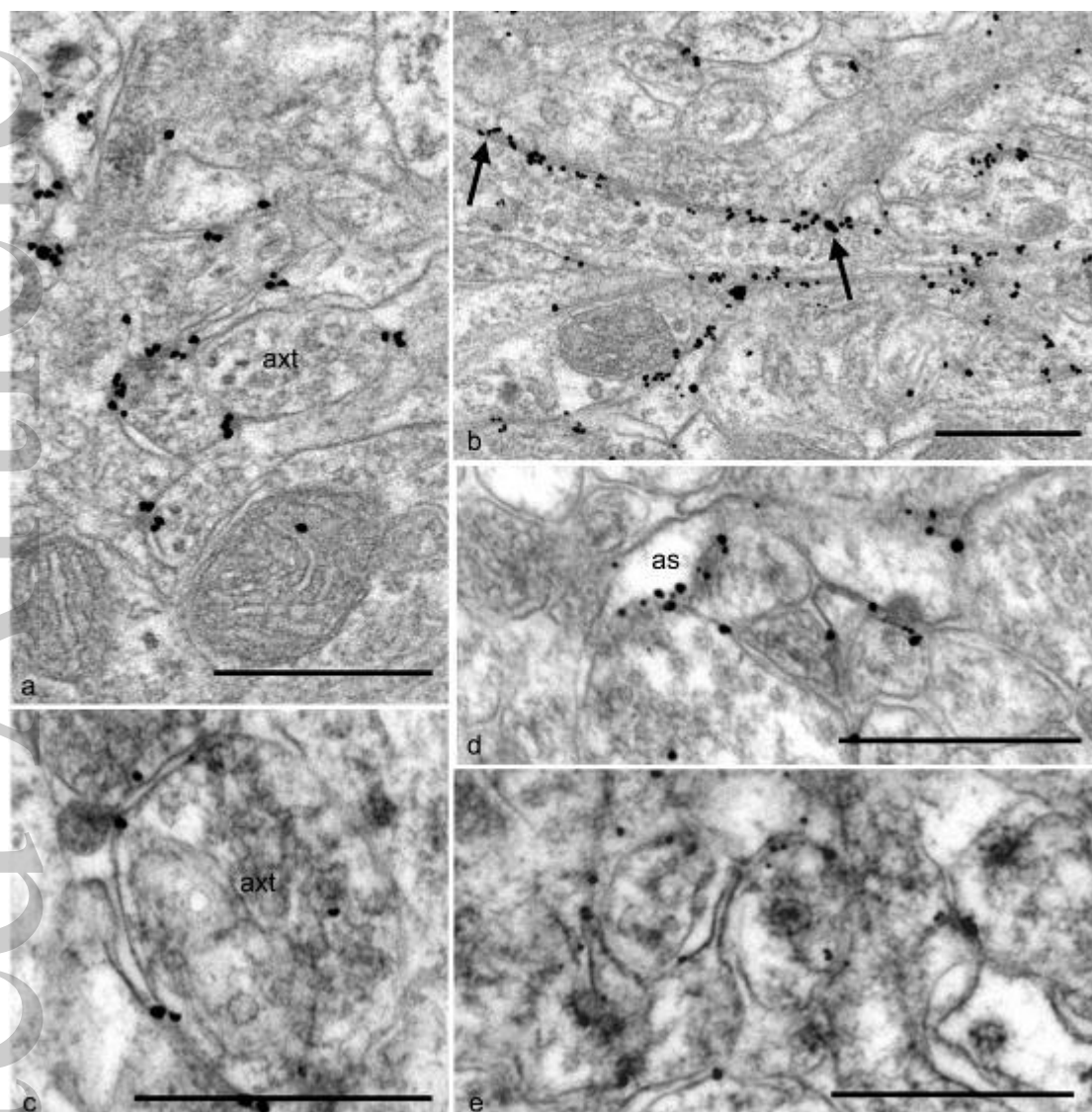


Figure 3

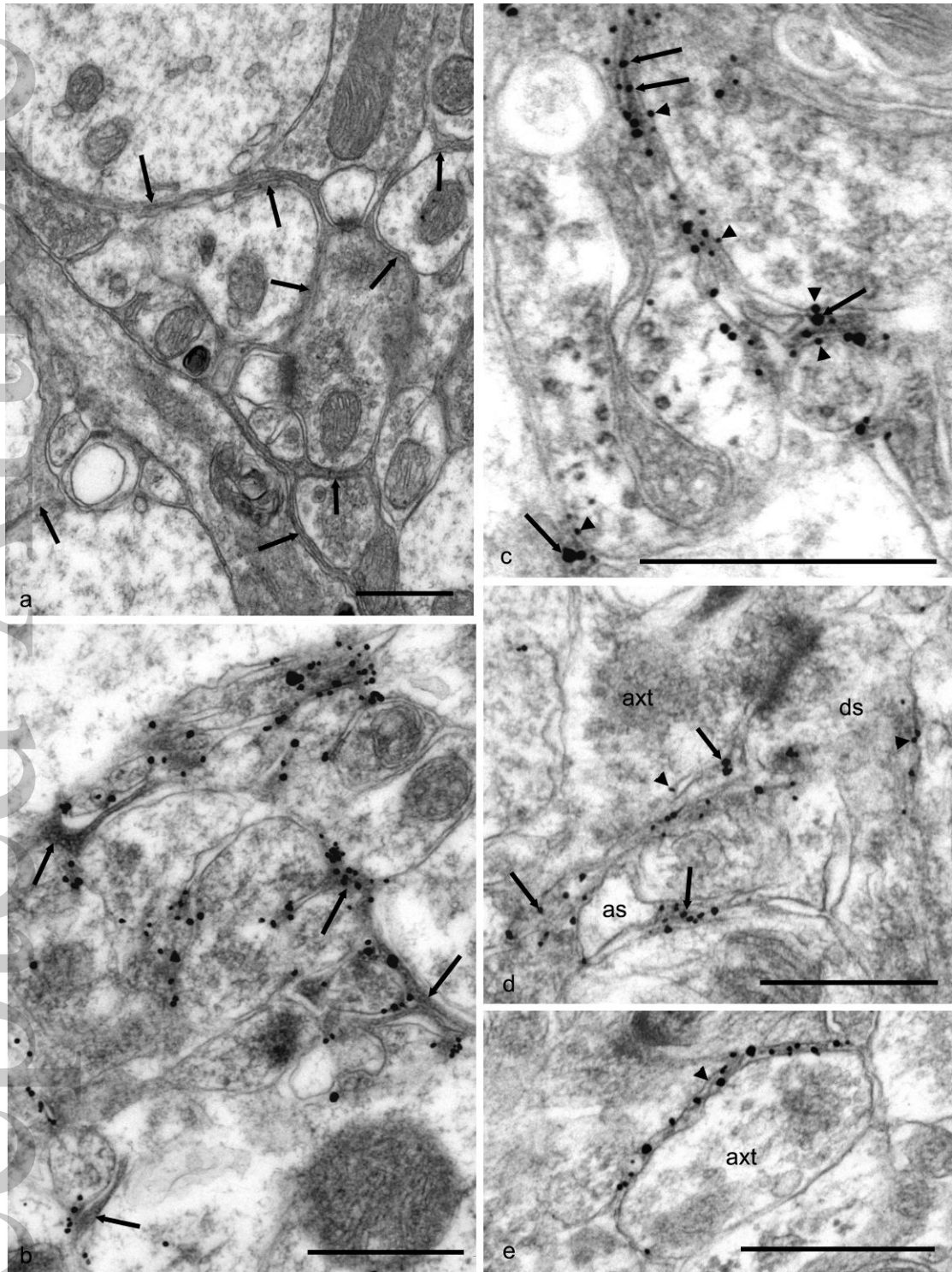


Figure 4

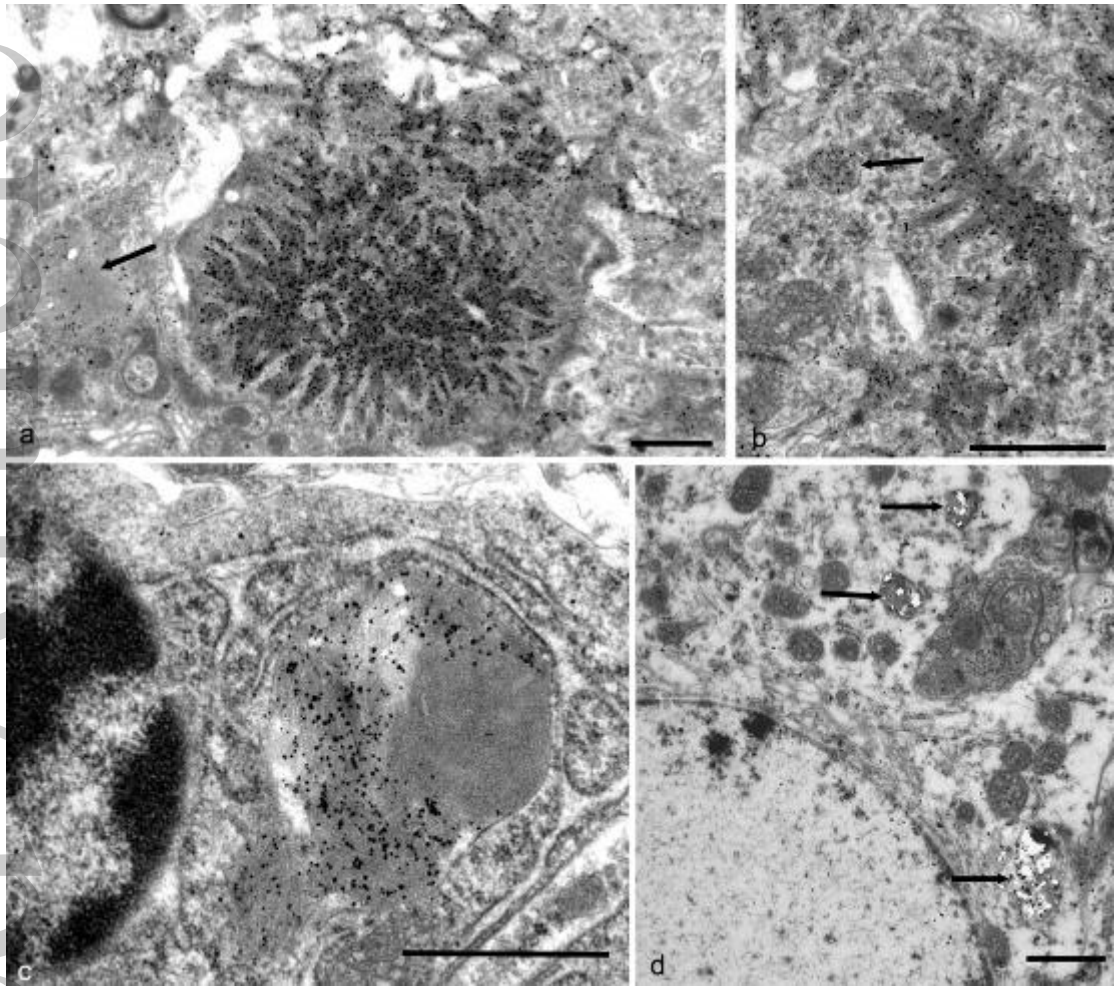


Figure 5